Exhibit 9

[CANCER RESEARCH 40, 3116–3117, September 1980]
0008-5472/80/0040-0000$02.00

Methylated Purines in Human Liver DNA after Probable Dimethylnitrosamine Poisoning[1]

Deborah C. Herron and Ronald C. Shank[2]

Departments of Community and Environmental Medicine [R. C. S.] and Medical Pharmacology and Therapeutics [D. C. H.], University of California, Irvine, California 92717

ABSTRACT

DNA, isolated from two samples of human liver obtained from a suspected dimethylnitrosamine poisoning, contained 1363 to 1373 μmol of 7-methylguanine per mol of guanine and 273 to 317 μmol of O^6-methylguanine per mol of guanine. Liver and kidney DNA obtained from unrelated cases contained no detectable methylated purines. From the DNA methylation levels, it is estimated that the dimethylnitrosamine-poisoning victim had been exposed to a dose of 20 mg or more of dimethylnitrosamine per kg of body weight. The results indicate for the first time that humans, like rodents, appear to activate dimethylnitrosamine metabolically to a strong methylating agent, resulting in methylation of liver DNA at both the 7- and O^6 positions of guanine.

INTRODUCTION

The hepatocarcinogenicity of DMN[3] has been demonstrated experimentally in 10 animal species (6), yet, in spite of known industrial and laboratory uses, no human cancers have been associated with exposures to this compound. It is now recognized that DMN is widely present in the environment in the food and air supplies and, under appropriate conditions, can form in the human stomach (8). Biochemical evidence suggests that this compound is a strong carcinogen in animals because of its metabolic activation to a highly reactive agent, which methylates target organ DNA at base-pairing sites such as the O^6 position of guanine (6). A recent case in forensic medicine in which 2 victims died of suspected DMN poisoning has now provided *in vivo* evidence that human beings seem to activate this nitrosamine metabolically in the same way, at least qualitatively, as do laboratory animals. Analysis of liver DNA prepared from one of the victims of suspected DMN poisoning indicates the presence of 7-methylguanine and O^6-methylguanine.

MATERIALS AND METHODS

Twelve frozen samples of human liver, kidney, and heart were received from the Center for Disease Control, Atlanta, Ga. and Poison Lab., Denver, Colo. Tissue analyses were performed without knowledge of the illness or cause of death. The phenolic extraction method of Kirby (4) as modified by Swann and Magee (10) was used to isolate and purify tissue DNA. Nucleic acid could not be isolated from 5 of the samples, which were severely autolyzed. DNA from the remaining 7 tissues was hydrolyzed in 0.1 M HCl (5 mg/ml) for 30 min at 70°, thus releasing all purines as free bases. Chromatographic separation of hydrolysates was carried out using a Partisil-10 strong cation-exchange column (inner diameter, 25 cm × 4.5 mm; Whatman, Inc., Clifton, N.J.) and 0.06 M ammonium phosphate (pH 2.0) at 2.0 ml/min (3). Elution of fluorescing bases was monitored using a 286-nm excitation wavelength with a 366-nm emission interference filter. Quantitation was achieved using electronic integration calibrated with standard solutions of authentic guanine, 7-methylguanine, and O^6-methylguanine.

RESULTS AND DISCUSSION

Only 2 DNA samples contained detectable amounts of methylated purines (Table 1). These samples were prepared from 2 liver specimens taken from a 23-year-old male victim of probable DMN poisoning.[4] The DNA samples from the other tissues were prepared from 2 cases of Reye's syndrome and one case of methyl bromide poisoning; none of these samples contained either 7-methylguanine or O^6-methylguanine. The amounts of these methylated bases found in the liver from the DMN victim were readily detectable and quantifiable (Chart 1).

This is the first report of the occurrence of O^6-methylguanine in human liver resulting from DMN exposure. Until recently, methylation of DNA following *in vivo* exposure to DMN was demonstrable only by using radioactively labeled carcinogen or large amounts of tissue, neither of which was available in the human poisoning considered here. The analytical method used in this study achieved high sensitivity by taking advantage of high-pressure liquid chromatography coupled with fluorescence detection (3).

An *in vitro* determination of metabolic activation of [^{14}C]DMN by rat and human liver slices was reported by Montesano and Magee (7). They found that human liver slices metabolized DMN at a rate such that 0.13% of the DNA guanine was methylated at position 7 in 1 hr compared to 0.17% of the DNA guanine in rat liver slices.

We have attempted to determine whether our data are consistent with an exposure to an acutely toxic dose of DMN. Craddock (1) has determined that a dose of 5 mg of DMN per kg of body weight results in the formation of approximately 1300 μmol of 7-methylguanine per mol of guanine in rat liver DNA 5 hr after administration of carcinogen p.o. If the human metabolizes DMN at only 68% of the rate at which the rat forms the active methylating agent as suggested by the study of Montesano and Magee (7) mentioned above, then the human dose could approximate 5 mg ÷ 0.68 or 7 mg/kg of body weight. Since the victim died 5 days after presumed exposure,

[1] This work was supported by the Environmental Toxicology Laboratories, College of Medicine, University of California, Irvine, Calif.

[2] To whom requests for reprints should be addressed.

[3] The abbreviation used is: DMN, dimethylnitrosamine.

Received January 7, 1980; accepted May 19, 1980.

[4] R. Kimbrough and S. Cooper, personal communication.

Table 1

Methylated purines in human DNA

DNA source	Cause of death	Methylated purines (μmol/mol guanine) 7-Methylguanine	Methylated purines (μmol/mol guanine) O^6-Methylguanine
Liver[a]	DMN poisoning	1363	273
Liver[a]	DMN poisoning	1373	317
Liver	MeBr[b] poisoning	ND[c]	ND
Kidney	MeBr poisoning	ND	ND
Liver[a]	Reye's syndrome	ND	ND
Liver[a]	Reye's syndrome	ND	ND
Liver	Reye's syndrome	ND	ND

[a] Two liver specimens analyzed from one victim.
[b] MeBr, methyl bromide.
[c] ND, not detected (limits of detection, 220 μmol 7-methylguanine per mol guanine and 8 μmol O^6-methylguanine per mol guanine).

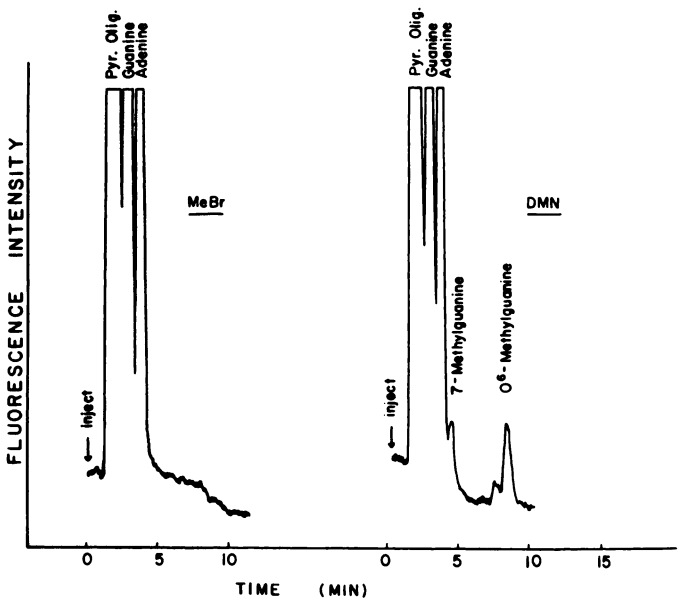

Chart 1. Elution profiles of liver DNA hydrolysate from victims of methyl bromide (*MeBr*) and DMN poisonings. DNA was hydrolyzed and fractionated to separate pyrimidine oligonucleotides (*Pyr. Olig.*), guanine, adenine, 7-methylguanine, and O^6-methylguanine by the liquid chromatographic method of Herron and Shank (3). Eluting bases were detected by fluorescence at a 286-nm excitation with a 366-nm emission interference filter. The unlabeled peak eluting before O^6-methylguanine has not been identified. The amount of 7-methylguanine in the liver of the victim of DMN poisoning is approximately 5 times greater than the amount of O^6-methylguanine. The eluting peak of O^6-methylguanine appears greater than that for 7-methylguanine because the relative fluorescence of O^6-methylguanine:7-methylguanine is 18:1 at a 286-nm excitation. (Redrawn from original.)

and since 7-methylguanine spontaneously depurinates from DNA (half-life, 24 to 48 hr), the human dose probably was considerably greater than 7 mg/kg of body weight. Pegg (9) has shown a dose-dependent relationship between DMN exposure and the O^6-methylguanine:7-methylguanine ratio in rat liver DNA. This ratio approaches 0.1 at about 20 mg of DMN per kg of body weight. The ratio in the human liver DNA reported here was 0.2; if extrapolation can be made from rat data, this provides additional evidence that the human exposure was probably greater than 20 mg/kg of body weight. The 50% lethal dose for DMN administered p.o. in the rat is 27 to 41 mg/kg of body weight (2); hence, the finding of the amounts of methylated purines in the human liver DNA reported here is consistent with an exposure to a level of DMN likely to be fatal.

In view of current theories of chemical carcinogenesis, the detection of methylated bases, particularly O^6-methylguanine, in human tissue after a probable exposure to DMN has important implications. Considerable attention has been focused on the presence and quantitative analysis of alkylated bases in DNA of animals treated with chemical carcinogens. Several attempts have been made to associate levels and sites of DNA alkylation with carcinogenicity. Although quantitatively a minor product, O^6-methylguanine may be more closely associated with carcinogenicity than the more frequently occurring 7-methylguanine (5). Both rats and humans, then, appear capable of metabolically activating DMN to a strong methylating agent, which interacts with the same sites in liver DNA in both species.

ACKNOWLEDGMENTS

We wish to thank Dr. Renate Kimbrough, Center for Disease Control, Atlanta, Ga. and Mr. Samuel Cooper, Deputy County Attorney, Omaha, Nebr. for supplying the tissue specimens.

REFERENCES

1. Craddock, V. M. Stability of deoxyribonucleic acid methylated in the intact animal by administration of dimethylnitrosamine. Rate of breakdown *in vivo* and *in vitro* at different dosages. Biochem. J., *111:* 497–502, 1969.
2. Heath, D. F., and Magee, P. N. Toxic properties of dialkylnitrosamines and some related compounds. Br. J. Ind. Med., *19:* 276–282, 1962.
3. Herron, D. C., and Shank, R. C. Quantitative high-pressure liquid chromatographic analysis of methylated purines in DNA of rats treated with chemical carcinogens. Anal. Biochem., *100:* 58–63, 1979.
4. Kirby, K. S. A new method for the isolation of deoxyribonucleic acids; evidence on the nature of bonds between deoxyribonucleic acid and protein. Biochem. J., *66:* 495–504, 1957.
5. Lawley, P. D. Carcinogenesis by alkylating agents. *In:* C. E. Searle (ed.), Chemical Carcinogens, ACS Monograph 173, pp. 83–244. Washington, D.C.: American Chemical Society, 1976.
6. Magee, P. N., Montesano, R., and Preussman, R. *N*-Nitroso compounds and related carcinogens. *In:* C. E. Searle (ed.), Chemical Carcinogens, ACS Monograph 173, pp. 491–625. Washington, D.C.: American Chemical Society, 1976.
7. Montesano, R., and Magee, P. N. Metabolism of dimethylnitrosamine by human liver slices *in vitro*. Nature (Lond.), *228:* 173–174, 1970.
8. National Research Council. Nitrates: An Environmental Assessment, pp. 435–484. Washington, D.C.: National Academy of Sciences, 1978.
9. Pegg, A. E. Alkylation of rat liver DNA by dimethylnitrosamine: effect of dosage on O^6-methylguanine levels. J. Natl. Cancer Inst., *58:* 681–687, 1977.
10. Swann, P. F., and Magee, P. N. Nitrosamine-induced carcinogenesis. The alkylation of nucleic acids of the rat by *N*-methyl-*N*-nitrosourea, dimethylnitrosamine, dimethylsulphate, and methyl methanesulphonate. Biochem. J., *110:* 39–47, 1968.